Acta Scientific Cancer Biology

Volume 1 Issue 2 July 2017

Mini Review

Oncolytic Potential of Tanapoxvirus for the Treatment of Melanoma

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Received: June 04, 2017; **Published:** September 25, 2017

Abstract

Melanoma is the deadliest skin cancer with significant morbidity and mortality. Oncolytic viruses (OVs), which are naturally occurring or genetically engineered viruses, have emerged as a class of novel therapeutic agents for melanoma treatment. At the confluence of virology, immunology and genetic engineering, OVs have been designed and optimized into anti-cancer agents using versatile strategies.

Tanapoxvirus (TPV), which possesses a large genome and causes only mild self-limiting diseases, appears as an ideal OV candidate. Here, we provide a summary of applying engineered TPVs as experimental melanoma immuno-virotherapy in our work, emphasizing the successful strategies used to achieve increased onco-specificity, oncolysis and immuno-reactivity.

Keywords: Tanapoxvirus, Oncolytic virotherapy, Melanoma, Interleukin-2, Neuregulin, Matrix metalloproteinase

Oncolytic virotherapy has recently been recognized as a promising therapeutic modality for cancer treatment and is perhaps the next major breakthrough in cancer immunotherapy. An oncolytic virus (OV) is a naturally occurring or genetically engineered virus that specifically or preferentially destroys cancer cells while sparing the normal tissues [1]. OVs not only cause tumor debulking directly via tumor cell infection and lysis, but also mediate the destruction of blood supply to tumors and induce both innate and adaptive anti-cancer immune responses. Since the first rationally designed oncolytic herpes simplex virus (HSV) proved its tumor regression capability and was reported in 1991 [2], the past two decades have witnessed the rapid and thriving development of OVs which demonstrate themselves to be excellent immunotherapies and boost the anti-tumor armament available to today's oncologists. The clinical milestone in OV development was the approval of two genetically engineered OVs for marketing as anti-cancer drugs. One is Oncorine, which is an oncolytic adenovirus approved by China Food and Drug Administration (FDA) for head and neck cancer and esophagus cancer in 2005 [3]. The other is T-Vec (Talimogene laherparepvec), which is an oncolytic HSV approved by FDA for melanoma therapy in the USA in October 2015 and subsequently in Europe in January 2016 [4]. With a variety of ingenious virus-engineering strategies and different ways to combine with other immunotherapies, the efficacy of OVs is anticipated to further improve for cancer treatment according to the stage and type of cancers.

Melanoma is a common skin cancer with significant morbidity. Although it accounts for approximately 3-5% of all skin cancers in US, it is responsible for nearly 80% of all skin-cancer related deaths [5-7]. Like most other types of cancer, melanoma arises from a combination of genetic and epigenetic abnormalities.

The genetic mutations such as over-activation of BRAF and Ras genes, which are found in respectively around 50% and 20% of metastatic melanomas, favor melanoma cell over-proliferation [8]. In addition, the loss of the expression of tumor-specific antigens and tumor-associated antigens, the lack of costimulatory signals for T cell activation, and the inefficient presentation of tumor antigens to antigen-presenting cells further contribute to melanoma survival and metastasis [9]. Conventional cytotoxic therapies are not always effective for melanoma. For example, the application of dacarbazine or vemurafenib resulted in less than 20% response rate in malignant melanoma patients [10]. In the continuous quest for development of new therapeutic agents for melanoma, OVs are highly promising treatment additions.

Each virus has a specific tropism that determines the types of cells infected and diseases induced. For example, human immunodeficiency virus (HIV) selectively infects T helper cells and causes acquired immunodeficiency syndrome [11]. Hepatitis B virus primarily replicates in hepatocytes and interferes with the functions of the liver [12]. Specificity for tumor cells instead of normal tis-

sues is the key for the safety of OV administration. To achieve this goal, attempts have been undertaken by using minimally virulent viruses or by genetically manipulating the viral genome. Tanapoxvirus (TPV), which belongs to the family Poxviridae (genus Yatapoxvirus), is a large virus containing double-stranded DNA genome (approximately 144 kbp) [13]. In contrast to some other viruses that cause severe disease, TPV is a relatively benign and geographically limited virus that only causes self-limiting and mild illness, resulting in fever, few skin lesions and lymph node enlargement in human and monkeys [14]. While TPV is only endemic in equatorial Africa, most of the global population is immunologically naïve to TPV, and no person-to-person transmission has yet been reported [14-16]. With a large genome for genetic modification and highly attenuated virulence, TPV can potentially serve as an ideal candidate for oncolytic virotherapy. In our work, we aimed to engineer TPV into effective OVs and expand the understanding of using OVs as melanoma immuno-virotherapy.

In our first study, we applied the strategy of adding anti-tumor efficacy to OV by arming OV with functional transgenes [17]. Interleukin (IL)-2, a T cell growth factor, plays a critical role in activating both the innate and adaptive immune systems. IL-2 is secreted by T cells and induces differentiation and development of thymic lymphocytes to become effector T cells [18]. In addition, IL-2 activates macrophages to become larger, more granular and conglomerated on the cancer cells with enhanced cytotoxicity [19]. Moreover, neutrophils become rapidly activated after exposure to IL-2 [20]. IL-2 transgene has been engineered into viruses such as reovirus and HSV for improving their oncolytic activity [21,22]. While studies have clearly demonstrated that OVs expressing IL-2 successfully increase the adaptive anti-tumor immune response including the proliferation of CD4+ and CD8+ T cells and result in effective tumor reduction [23], few studies have explored the involvement of the innate immune response elicited by IL-2 expressing OVs. In addition, studies regarding the role of IL-2 in virus replication have generated conflicting results. While some studies suggest that IL-2 plays a protective role against virus infection [22], others suggest that IL-2 likely supports viral infection [24]. Therefore, in this study we aimed to investigate (a) if the viral lysis together with the innate anti-tumor immune response mediated by the IL-2 expressing OVs is therapeutically sufficient for melanoma elimination in vivo and (b) the exact effect of IL-2 on virus replication, which is of great interest but still remains unclear.

Deletion of viral thymidine kinase gene (*TK*) has widely been used for attenuating OV replication in normal tissues and enhancing tumor selectivity, as tumor tissues which overly produce TK are able to complement this gene deletion and support virus replication [4]. In this study, a recombinant TPV expressing mouse IL-2 transgene, replacing the viral *TK* gene (*66R*), was generated and referred to as TPVΔ66R/mIL-2. In cell culture, expression of IL-2 attenuated virus replication of not only TPVΔ66R/mIL-2, but also TPVGFP (TPV expressing green fluorescence protein) when co-infecting with TPVΔ66R/mIL-2. Further, the neutralization of either IL-2 or interferons (IFNs) in the cell culture exerted no significant effect in boosting TPVΔ66R/mIL-2 replication, which demonstrates that IL-2 inhibits virus replication through intracellular components and without activating the IFN-signalling pathway. The anti-tumor potential of TPVΔ66R/mIL-2 was studied in thymic nude mice (T cell deficient animals) carrying human melanoma xenografts. Introduction of mIL-2 into TPV remarkably increased its anti-tumor activity, resulting in a more significant tumor regression than with wild-type (wt) TPV and TPVΔ66R. On average, the tumor volume increased by 4.49%/day in the mice with mock treatment but only by 0.06%/day in the TPVΔ66R/mIL-2-treated mice. In contrast, the mean tumor growth rate in the wtTPV- or TPVΔ66R- treated mice was not significantly lower than that in the mice treated with mock injection.

Histopathological observations showed that treatment with TPVΔ66R/mIL-2 resulted in significantly increased accumulation of inflammatory cells such as neutrophils, lymphocytes and macrophages, in comparison to treatment with the other TPVs. The host-derived cells of mononuclear form, potentially both lymphocytes and macrophages, were more abundantly present in tumors treated with TPVΔ66R/mIL-2. However, mononuclear cells that were F4/80 negative were only present in the TPVΔ66R/mIL-2-treated tumors and were not seen in any of the other treatment groups. While anti-CD3 staining showed no positivity in tumor samples of all treatment groups, indicating the absence of T cell response, anti-Ki67 staining showed more extensive tumor cell degeneration and greater amount of degenerating melanin present in TPVΔ66R/mIL-2-treated tumors. These data provide compelling evidence that IL-2 induces a host anti-tumor innate immunity for tumor regression in the absence of mature T cells. Taken together, the significance of this study lies in: (a) it suggests that TPVΔ66R/mIL-2 is a promising immuno-virotherapy for melanoma; (b) it contributes to understanding of the function of IL-2 in virus replication; and (c) it strongly suggests that the incorporation of IL-2 gene into viral genome significantly increases virus-mediated anti-tumor activity without mature T cells, which is meaningful and supportive for investigating the administration of IL-2 expressing OV in patients carrying a T cell deficiency.

In our second study, we aimed to engineer oncolytic TPV by manipulating the viral immuno-modulators [25], which are the proteins encoded and expressed by many viruses to modulate the innate and adaptive immune responses [26]. Neuregulin's (NRGs), a family of epidermal growth factors (EGFs), are the ligands for ErbB3 and ErbB4 receptors which belong to ErbB family of receptor tyrosine kinases [27]. NRGs are originally implicated in the development of the nervous system and play multiple essential roles in embryonic endocardium development, Schwann cell and oligodendrocyte differentiation [28]. However, more recent studies have shown that by binding to ErbB3 and ErbB4 receptors, NRG stimulates melanocytes to gain increased proliferation and invasion, altered morphology and enhanced expression of progression and metastasis genes [29-31]. Our previous studies have shown that TPV-encoded protein (15L) biologically mimics NRG and possesses the ability of binding heparin and phosphorylating NRG re-

ceptors [28]. In this work, we show that TPV-15L protein promotes melanoma cell growth, and the proliferative efficacy of TPV-15L is indistinguishable from NRG. In light of this, we genetically engineered TPV with 15L gene deletion and with or without the deletion of *66R* gene that encodes TK, thus generating TPVΔ15LΔ66R and TPVΔ15L recombinant viruses, respectively. TPVΔ15L showed replication ability similar to that of wtTPV, indicating that *TPV-15L* gene is nonessential for virus replication.

However, TPVΔ15LΔ66R replicated less efficiently compared to wtTPV, TPVΔ66R and TPVΔ15L, suggesting that deletion of *66R* gene but not *15L* gene adversely affects the viral replication. We further studied whether deletion of *TPV-15L* gene (encoding NRG mimicking protein) would abolish the growth-promoting effect of TPV-15L protein on human melanoma cells and enhance oncolytic efficacy of TPV in melanoma-bearing animal model. Tumor xenografts were established using human melanoma SK-MEL-3 cells on nude mice, which were treated with intratumoral injection of wtTPV, or one of the TPV recombinants (TPVΔ15L, TPVΔ66R or TPVΔ15LΔ66R). Our results demonstrated that TPVΔ15L exhibited a more robust tumor reduction compared to wtTPV, TPVΔ66R and TPVΔ15LΔ66R. TPVΔ15LΔ66R was less effective in regressing melanoma tumors, which is possibly due to its slower replication. The greater tumor regression efficacy of TPVΔ15L compared with wtTPV and other TPV recombinants suggests that the reduced melanoma proliferation possibly relates to the absence of tumor-enhancing properties caused by TPV-15L protein. Taken together, this study is meaningful as it supports that TPVΔ15L can be a promising oncolytic virotherapy for melanoma, and suggests that deletion of viral genes encoding NRG-like proteins or EGF-like growth factors is potentially an effective strategy to be used in genetic engineering of OVs for melanoma. Moreover, this study explores the virological, neurological and immunological aspects of TPV, broadening the understanding of using TPV as oncolytic virotherapy.

In the third study, we aimed to explore the impact of TPV on tumor microenvironment and improve TPV's oncolytic efficacy by manipulating its immuno-modulatory activities [32]. Metastasis is the primary cause of the mortality of melanoma patients and is a complex process. It includes the invasion of the tumor cells to the surrounding tissues and basement membranes, the penetration into the blood and lymphatic vessels, and the re-penetration into other organs to form detectable tumors [33]. Matrix metalloproteinases (MMPs), which are involved in degradation of extracellular matrix (ECM) in the tumor microenvironment, are critical regulators in tumor progression, metastasis and angiogenesis [34,35].

MMP-9, also known as gelatinase B, degrades type IV and V collagens and gelatins, which are essential components of the ECM [36]. Increased MMP-9 expression is correlated with many diseases and pathological abnormalities including melanoma [33]. Expression of MMP-9 has been shown to enhance melanoma growth and metastasis, such as lung colonization [37]. The infections of some viruses, such as respiratory syncytial virus, influenza virus and HIV, and their accompanying pathogenesis are often associated with increased production of MMP-9 [38,39]. For example, infection of macrophages with HIV results in enhanced level of MMP-9 production [40]. Although induction of MMP-9 is frequently observed during virus infection and elevated MMP-9 levels are often correlated with disease severity, the functional role of MMP-9 in viral replication and infection still remains controversial. While the presence of MMP-9 enhances the replication of some viruses such as HSV [41], an anti-viral effect of MMP-9 has also been observed in the replication of others [42]. In this study, we sought to investigate the interplay between MMP-9 expression and TPV replication in melanoma cells and to determine whether oncolytic efficacy of TPV could potentially be enhanced by manipulation of MMP-9 expression. Here we report that MMP-9 expression is significantly enhanced in human melanoma SK-MEL-3 cells infected with wtTPV and TPV recombinants (TPVΔ15L and TPVΔ66R), relative to control samples that were mock infected. Moreover, the MMP-9 expression was even more remarkable in TPVΔ15L-infected cells, compared with that in wtTPV- and TPVΔ66R-infected cells. While MMP-2, the other gelatinase, has been shown to degrade type IV collagen and promote cancer invasion and metastasis [43], we have been unable to detect MMP-2 in SK-MEL-3 cells, either infected or uninfected. However, elevated tumor growth factor-β was observed in SK-MEL-3 cells infected with TPVs compared with that in uninfected cells. While previous studies show that the role of MMP-9 in virus replication varies between viruses, we show that MMP-9 exerts an anti-viral effect on TPV replication and plays a protective role in TPV-infected melanoma cells in vitro. For example, at 0.1 multiplicity of infection (MOI) upon treatment with 10 μg/ml MMP-9, the yield of TPV replication was ten-fold decreased and the percentage of infected cell survival was approximately two-fold greater at 96 hours post infection (hpi), compared to that with no MMP-9 treatment (mock). At 5 MOI upon treatment with 10 μg/ml MMP-9, the yield of virus replication was three-fold decreased and the percentage of infected cell survival was approximately two-fold increased, relative to that with mock treatment at 96 hpi.

In contrast, the neutralization of MMP-9 in human melanoma SK-MEL-3 cells remarkably enhances the TPV infection and leads to a significant reduction in cell survival. Taken together, this study contributes to understanding of the role played by MMP-9 in TPV infectivity and provides more insights for using TPV as cancer virotherapy in future studies. Since TPV has shown substantial oncolytic efficacy in promoting melanoma tumor regression in animal models, identifying mechanisms that suppress MMP-9 expression upon TPV infection can potentially improve its use as a melanoma virotherapy. Another remarkable aspect of this study is that it suggests attention should be paid to the anti-viral activity of some MMPs including MMP-9, as strategies have been designed to incorporate MMPs into OVs to assist the virus replication and spread by degrading ECM components in some studies.

To summarize, the development of OVs has entered an exciting age in which OVs are being actively translated into therapeutic options for cancers including melanoma. Challenges in this field are to select the "winners", which possess the maximal viral oncolysis and anti-tumor immunity and the best clinical outcomes [44]. For some virus platforms such as adenovirus and reovirus, the pre-existing anti-viral immunity developed by the host immune system potentially inactivates the virus and impedes the efficacy of systemic administration. In addition, the anti-viral immune response of the host makes it uncertain for OVs to be "one shot" therapeutic agent and make a virus less effective during successive treatment cycles.

While an effective virus-specific immuno-tolerance strategy is highly desirable to be integrated into OVs, presently it may be necessary to have a set of antigenically distinct OVs for serial injection to avoid the problem. Moreover, safety profile of a virus determines its feasibility as a virotherapy. Considering these factors, TPV represents an ideal OV platform and addition to OV bank due to benefits that include a large genome, antigenic distinction and no reported human-to-human transmission [25]. In this work, we focus on applying TPV as a melanoma immuno-virotherapy and describe three studies in which different approaches have been used in the hope of enhancing TPV's oncolytic efficacy. These include (a) arming OV with immuno-stimulatory cytokines (incorporation of IL-2), (b) engineering viral immuno-modulators (deletion of NRG mimicking gene), and (c) manipulating components in the tumor microenvironment (neutralization of MMP-9). In the first study, our data indicate that IL-2 plays a protective role in inhibiting TPV replication and that TPVΔ66R/mIL-2 is robust in eliciting host innate immunity for melanoma tumor regression in addition to the direct viral cytolysis.

Our results suggest that IL-2 expressing OVs might be therapeutically effective for melanoma elimination without T cells, therefore serving as an appealing treatment option for melanoma patients with T cell suppression/deficiencies. In the second study, we are among the first to show that TPV-15L gene product exhibits a similar growth promoting effect on human melanoma cells as NRG originally referred as a functional protein in nervous system development. Our results show that deletion of TPV-15L gene product which facilitates the growth of melanoma cells can be an effective strategy to enhance the oncolytic potential of TPV for melanoma treatment. In the third study, we are among the first to show that TPV infection correlates to the induction of MMP-9 in melanoma cells. Our results indicate that MMP-9 possesses an anti-viral activity on TPV replication, and suggest a combination of MMP-9 neutralization and TPV for oncolytic efficacy improvement.

Conflict of Interest

Authors declare that they have no conflict of interest.

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